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Long-term Effects of Neonatal Exposure to Hydroxylated Polychlorinated Biphenyls in the BALB/cCrgl Mouse

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ABBREVIATIONS: 17 beta-estradiol (E2), 2’4’6’-trichloro-4-biphenylol (OH-PCB 30), 2’3’4’5’-tetrachloro-4-biphenylol (OH-PCB 61), aryl hydrocarbon receptor (AhR), cervicovaginal (CV), day of vaginal opening (DVO), diethylstilbestrol (DES), estrogen receptor (ER), hydroxylated polychlorinated biphenyls (OH-PCBs), mouse mammary tumor virus (MMTV).

Outline of manuscript section header

ABSTRACT

The neonatal mouse model has been a valuable tool in determining the long-term effects of early exposure to estrogenic agents in mammals. Using this model, we compared the effects of 2'4'6'-trichloro-4-biphenylol (OH-PCB 30) and 2'3'4'5'-tetrachloro-4-biphenylol (OH-PCB 61) as prototype estrogenic hydroxylated PCBs (OH-PCBs) since they are reported to exhibit relatively high estrogenic activity both *in vivo* and *in vitro*. The purpose of this study was to examine the relationship between estrogenicity and carcinogenicity of OH-PCB congeners. The OH-PCBs were tested individually and in combination to determine whether effects of combined OH-PCBs differed from those of these OH-PCBs alone. We evaluated the long-term effects of neonatal exposure to OH-PCBs with treatment doses that were based on the reported binding affinity of specific OH-PCB congeners to the estrogen receptor alpha. BALB/cCrgl female mice were treated within 16 hr after birth by subcutaneous injections every 24 hr, for 5 days. The OH-PCB 30 [200 μg/day] or 17 beta-estradiol (5 μg/day) showed similar increased incidences of cervicovaginal (CV) tract carcinomas (43 % and 47 %, respectively). In addition, when mice were treated with OH-PCBs as a mixture, a change in the type of CV tract tumor was observed, shifting from predominantly squamous cell carcinomas to adenosquamous cell carcinoma. From our results, we concluded that the individual OH-PCBs tested were estrogenic and tumorigenic in mice when exposed during development of the reproductive tract. These data support the hypothesis that mixtures may act differently and unexpectedly than as individual compounds.

INTRODUCTION

Evidence that estrogen acts as a gynecological carcinogen comes from cases of adenocarcinoma and non-neoplastic abnormalities of the genital tract in females who had been exposed to diethylstilbestrol (DES) *in utero* (Herbst et al. 1971; Robboy et al. 1977). The subsequent cases of cancer and other gynecological abnormalities in females exposed to DES *in utero* helped to establish the paradigm that a developing fetus is sensitive to compounds tolerated by adults. This paradigm led researchers to reexamine the potential effects of endocrine-disrupting chemicals in human and wildlife species (Gray 1998; Santodonato 1997; Semenza et al. 1997; Zou and Fingerman 1997).

In mice, neonatal exposure to potent natural and synthetic estrogens results in the development of cervicovaginal tumors, some of which resemble tumors in human females exposed to DES *in utero* (Bern et al. 1975; Bern and Talamantes 1981). Most significantly, these tumors in the mouse model like those in women transplacentally exposed to DES, are dependent on the dose and time of exposure to the estrogen. Correlation of estrogenicity of DES with carcinogenicity has been demonstrated in the mouse uterus, but requires an endogenous source of estrogen for both tumor initiation and progression (Newbold et al. 1990). 17 alpha-estradiol, is a natural estrogen that binds weakly to the estrogen receptor. In mice, exposure to17 alpha-estradiol during a critical period of reproductive tract development leads to subsequent gynecological malignancies (Hajek et al. 1997). These studies exemplify that various abnormalities in long-term studies are dependent on ***when*** mammals are exposed to a natural or synthetic estrogen.

While there are a large number of known estrogenic chemicals, we were interested specifically in estrogenic hydroxylated polychlorinated biphenyls (OH-PCBs), because the role

they play in breast cancer is controversial and uncertain (Adami et al. 1995; Aschengrau et al. 1998; Krieger et al. 1994). PCBs belong to a class of organochlorine synthetic chemicals that have up to 209 congeners or configurations possible, depending on the number and location of chlorines on the molecule. These PCBs vary in the number of chlorine atoms present, which ranges from 1 - 10, as well as their position on the two benzene rings. The relative toxicity of PCBs depends upon chemical characteristics such as chlorination, hydrophobicity and planarity (Brouwer et al. 1999). The biological activity of PCBs is generally classified as dioxin-like or non-dioxin-like depending on their mechanism of action. Dioxin-like compounds assume a coplanar configuration with chlorine atoms on the *meta* or *para* benzene position and have a high binding affinity to the aryl hydrocarbon receptor (AhR). Through activation of the AhR they elicit dioxin-like biochemical and toxic responses. Non-dioxin-like chemicals assume a non-coplanar configuration with chlorine atoms on the *ortho* benzene position and bind with variable affinities to steroid hormone receptors. Certain PCBs found in the environment have been shown to be are estrogenic, for example Hansen *et al.,* (1995) demonstrated that landfill-associated extracts containing PCBs are uterotropic in prepubertal rats. PCB congeners that are capable of binding to the estrogen receptor (ER) and can induce the following estrogen-related effects in rodents: increased uterine wet weight, increased glycogen content, prolonged estrous cycle, and proto-oncogene expression (Ecobichion and MacKenzie 1974; Gellert 1978; Korach et al. 1988). 4-Hydroxylated PCB (OH-PCB) are the major metabolites of PCBs and are found in human and wildlife blood and appear to persist and bioaccumulate (Bergman et al. 1994; Hovander et al. 2002; Li et al. 2003). They are formed by an arene oxide intermediate catalyzed by Phase I cytochrome P450s. However, the toxicological impact of the OH-PCB and their adverse effect in human are not well characterized. The placental transfer of OH-PCBs has been recent established

(Soechitram et al. 2004) suggesting that these PCB metabolites could have adverse effects during developmental exposure. OH-PCBs have been shown to be antiestrogenic, estrogenic and bind to the estrogen receptor, bind to the thyroid hormone receptor and are in general endocrine disrupting chemicals (Arulmozhiraja et al. 2005; Connor et al. 1997; Kitamura et al. 2005; Korach et al. 1988).

The goal of this study was to determine if neonatal exposure to the estrogenic chemicals 2'4'6'-trichloro-4-biphenylol (OH-PCB 30) and 2'3'4'5'-tetrachloro-4-biphenylol (OH-PCB 61) results in carcinogenicity. The positions of the chlorines for these two PCBs are indicated in Figure 1. The OH-PCBs are the 4-hydroxylated metabolites of parent PCB 30 and PCB 61. These PCB congeners were chosen because they have known estrogenic activity and their binding affinity to the estrogen receptor is reported in the literature (Table 1). Investigations of early life-stage exposure to polychlorinated biphenyls (PCBs) are warranted since these organochlorine chemicals and their metabolites readily cross the placenta to the fetus in both humans and rodents and are transferred through the breast milk to the newborn (Ando 1978; Ando et al. 1985; Ando et al. 1986). There is a growing database on developmental effects for endocrine disrupting chemicals with multiple endpoints including cancer. In this study we examined the neonatal effects of OH-PCBs. While the specific OH-PCB used investigated in this study may not occur in the environment they are sound prototypes for estrogenic hydroxylated PCBs that bind to estrogen receptor alpha and elicit estrogen-mediated responses.

MATERIALS AND METHODS

Chemicals. All chemicals were of the highest grade available. 17 beta-estradiol (E2) was purchased from Sigma Chemical Co. (St Louis, MO). Both 2'4'6'-trichloro-4-biphenylol (OH-PCB 30) and 2',3'4',5'-tetrachloro-4-biphenylol (OH-PCB 61) were kindly and generously provided by Dr. Stephen Safe (Texas A&M University, College Station, TX). These OH-PCBs were synthesized and purity confirmed as described previously (Safe et al. 1995). For this study, E2 and the OH-PCBs were dissolved in 1 ml of 100 % ethanol and warmed to dissolve the chemical. Sesame oil was added to obtain the desired concentrations for 20 µL subcutaneous injections. Ethanol was then evaporated using nitrogen gas and keeping the solution warm to prevent recrystallization. OH-PCB doses used in this study are based on their reported respective binding affinity to the alpha estrogen receptor. E2 at 5 µg/day was used as a predictive dose since the frequency of CV tumors in BALB/cCrgl mice neonatally E2 exposed is approximately 50 % (Jones and Bern 1979). To test for interactive effects, doses were selected using the high dose of OH-PCB 30 as a basis of comparison, since it has a higher binding affinity to the estrogen receptor alpha.

Animals. Mice were handled according to the Principles of Laboratory Care (NIH Publication No. 86-23), and the institutional animal care and use committee approved all procedures performed on animals. Adult mice were fed Purina Rodent Chow 5001. Pregnant female BALB/cCrgl, mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). The inbred BALB/cCrgl strain was used since it has a low mammary tumor incidence and its response to E2 treatment during neonatal development is well documented. Beginning within 16 hours after birth, female pups were pooled from several litters and distributed 4-5 pups per mother per cage.

Each cage was then given 5 daily subcutaneous injections with either 20 µl of sesame oil alone, 5 µg E2, 2.5 µg E2 plus 100 µg OH-PCB 30, 20 µg OH-PCB 30, 200 µg OH-PCB 30, 40 µg OH-PCB 61, 400 µg OH-PCB 61, 10 µg OH-PCB 30 plus 10 µg OH-PCB 61, or 100 µg OH-PCB 30 plus 100 µg OH-PCB 61 (Table 2). Weaning of animals was at 21 days of age. Mice were examined daily for premature vaginal opening for the first 35 days from birth, and checked monthly with blunt forceps to detect concretions. When concretions were found they were removed. All mice that survived to 20 months of age were sacrificed by CO_2 fixation. Tissues were dissected and fixed in 10 % buffered formalin for at least 24 hours before being embedded in paraffin. Paraffin-embedded blocks were serially sectioned and stained with hematoxylin and eosin.

Statistical Analyses. One-way analysis of variance (ANOVA) was used to assess differences in body weight, uterine weight, and vaginal opening. Pair-wise comparisons of each experimental group versus sesame oil control were made by Tukey-HSD tests. Survival comparisons were made by Wilcoxon rank sum tests. The proportions of animals with malignant tumors were compared by Fishers exact tests. Animals dying prior to the appearance of the first tumor were excluded from the analysis.

RESULTS

Gross Observations

A biological index of sexual maturity can be visually assessed by day of vaginal opening (DVO). The DVO was significantly shorter in mice given E2 alone, E2 plus OH-PCB 30 OH-PCB 30 (200 μg), OH-PCB 61 (40 and 400 μg), and the mixture OH-PCB 30/61 (100/100 μg) (Table 2). There was a dose-dependent effect with the higher dose yielding the shortest DVO. The lower doses of OH-PCBs had a DVO similar to control mice. Body weight was significantly decreased in mice given 5 μg E2. Mortality was increased in mice given OH-PCB at high doses ($P < 0.05$) (Table 2).

Tumor Incidence

Tumor incidences are summarized in Table 3. The only tumor seen in control mice was one malignant lymphoma. The incidence of malignant tumors was significantly greater in all groups exposed to estradiol and/or PCB than in controls. Among mice given E2 alone, incidences of cervicovaginal (CV) tract carcinomas was 43 % (16/37), or for any tumor 49 % (18/37), note some mice had more than one type of tumor detected). Other tumors that are commonly observed in mice neonatally treated with E2 were detected and include cholangiocarcinoma of the gallbladder, and granulosa cell tumor. The E2 treated mice also had one incidence of bronchoalveolar adenoma of the lung. Among mice given E2 plus OH-PCB 30, incidences of CV tract carcinomas 47 % (9/19), granulosa cell tumors 15 % (3/19), were significantly increased. In addition there was one reticulum cell sarcoma detected in mice treated with E2 plus OH-PCB 30.

In mice given the high dose of OH-PCB 30, the incidence of CV tract carcinomas was 45 % (10/22), granulosa cell tumor was 14 % (3/22), and one mouse was detected with

cholangiocarcinoma. Low dose of OH-PCB 30 given to mice neonatally resulted in: 6 % (2/33), CV tract carcinomas, 15 % (5/33), incidence of mammary gland adenocarcinoma and 9 % (3/33), bronchoalveolar adenoma/carcinoma.

In mice given OH-PCB 61 [400 μg] neonatally, the incidence of CV tract carcinomas was 20 % (5/24), mammary gland adenocarcinoma 4 % (1/24), and hemangiosarcoma 8 % (2/24). There was one animal from all treatment groups with hepatocellular carcinoma, this was observed in mice treated with OH-PCB 61 [400 μg]. In mice treated with the low dose of OH-PCB 61 [40 μg], the incidence for the following neoplasms were the following: CV tract carcinomas 13 % (4/30), granulosa cell tumor 10 % (3/30), mammary gland tumors 13% (4/30), and hemangiosarcomas 7 % (1/30). Mice given the mixture OH-PCB 30/61[200 μg] the incidence of neoplasms detected were as follows: CV tract carcinomas 38 % (8/21), granulosa cell tumor 10 % (2/21), malignant lymphomas 10 % (2/21), and bronchoalveolar carcinoma 5 % (1/21). Incidence rates for mice treated with OH-PCB 30/61 [20 μg] were: CV tract carcinomas 8 % (3/36), granulosa cell tumor 3 % (1/36), and mammary gland carcinomas 8 % (3/36).

Interactive Effects of Chemical Mixtures

The two types of tumors detected in groups administered estrogenic compounds alone and in combination were compared with Fishers exact test (Table 4). No detectable differences in the overall incidence of CV tract tumors were observed. However, there was a difference though in the relative distributions of tumor types. In animals treated with E2, 8 % (3/37), or the high dose of OH-PCB 30, 14 % (3/22), a significant difference between the combined incidences of CV tract adenosquamous cell carcinoma was observed compared to that of animals treated with E2 /OH-PCB 30, 32 % (6/19), as determined using a Fishers Exact tests. Although not

statistically significant, there appeared to be a trend for an increased incidence of CV tract development of adenosquamous cell carcinoma versus squamous cell carcinoma when comparing the combined incidence of OH-PCB 30, 14 % (3/22), and OH-PCB 61, 8 % (2/20), to that of OH-PCB 30/61, 24 % (5/21).

DISCUSSION

In this study, we used the DES neonatal mouse model to evaluate the tumorigenic effects of estrogenic OH-PCBs. The results show that the production of CV tract tumors occurred to a similar degree between E2 [5 µg] 43 % (16/37), and OH-PCB 30 [200 µg] 47 % (9/19). There were a rather large number of different tumors detected in this study but the tumors with the highest frequency were the CV tract tumors (Table 3). Neonatal OH-PCB treatment caused the induction of CV tract tumors in this study. A limitation of this study was the number of doses used, but there did appear to be a pattern of increased CV-tract tumors with the higher doses. These data strongly support the theory that relatively weak estrogens can induce tumors in mice when exposure occurs during a critical time of development (Hajek et al. 1997).

The neonatal mouse model has been extensively studied for over four decades and proven extremely valuable in assessing human *in utero* exposure to diethylstilbestrol (DES). The defined period for causation of genital tract tumors by natural (17 alpha-estradiol and E2) and synthetic estrogens (*e.g.*, DES) occurs during the development of the reproductive tract in both humans and rodents (Hajek et al. 1997). The use of the neonatal mouse model was necessary because, unlike findings in adult-treated rodents (Liehr et al. 1986), an apparent correlation between estrogenicity and carcinogenicity exists in neonatally treated rodents (Newbold et al. 1990; Newbold et al. 1997). In addition, species specific E2-mediated tumor induction occurs in different strains of mice. For example, outbred female CD-1 mice are susceptible to uterine tumors, and inbred BALB/cCrgl mice are hormonally susceptible to cervicovaginal (CV) tract tumors (Jones and Bern 1977). E2-mediated tumor induction is also age-dependant, dose-related and, most importantly, occurs in a tissue-dependent manner (Newbold et al. 1990).

Experiments were aimed at determining a relationship between estrogenicity and carcinogenicity for estrogenic PCBs. The first indication of the estrogenicity of E2 and/or OH-PCBs in this study was premature vaginal opening (Table 2). OH-PCBs tested alone or in combination facilitated premature vaginal opening in a timeframe similar to that of E2. Both OH-PCB 30 and OH-PCB 61 have also tested positive for *in vivo* estrogenicity in juvenile fish and mice (Carlson and Williams 2001; Korach et al. 1988). Like other studies testing interactions we only found additive effects from the combined chemicals (Carlson and Williams 2001; Ramamoorthy et al. 1997). We found that the highest mortality rates were seen in mice treated with high doses of OH-PCBs indicating that neonatal exposure to PCBs has a chronic toxic effect since the majority of those died close to twelve months. Some of the chronic carcinogenic effects attributed to OH-PCB exposure in this study were similar to those known for E2**,** but others such as tumor formation in other organs besides the CV tract, were not. Thus, the tumors seen in E2-treated mice reflect the species-specific E2 mediated tumor susceptibility of BALB/cCrgl mice. In contrast to findings in the literature that mixtures of PCBs promote hepatocellular carcinoma, (Dutch Expert Committee on Occupational 1995; Mayes et al. 1998; Sleight 1985), a variety of malignant tumors were identified in the OH-PCB treated mice, but only one mouse developed a hepatocellular carcinoma, thus the mechanisms are likely to be very different.

The mammary gland carcinomas incidence was significantly increased to 13 % (4/30) in mice treated with OH-PCB 61 [40 µg]. There were also mammary gland tumors detected in mice treated with E2, 3% (1/37), OH-PCB 61 [400 µg] 13 % (4/30), OH-PCB 30 [20 µg] 15 % (5/33), and OH-PCB 30/61 [20 µg] 8 % (3/36). While there are several studies published that support the idea that developmental exposure to PCBs may lead to an increase in breast cancer

(Birnbaum and Fenton 2003; Desaulniers et al. 2001; Mayes et al. 1998), the results from this study are striking that there was any increased number of mammary tumors detected. Historically BALB/cCrgl mice do not develop mammary gland tumors (Dunn and Green 1963; Mori et al. 1976). A clear dose-dependant increase in mammary gland tumor responses was not found since there were no further increases in mammary gland tumors in the low and high dose OH-PCB 61 mice. No mammary gland tumors were detected in the high dose OH-PCB 30 mice as opposed to the low dose. Why this effect occurred is likely to be the result of the increased mortality in high dose groups (Table 2). Unfortunately, no dissections or histological analysis occurred if animals died on weekends or nighttime. In addition, the mammary glands were not dissected out from control animals and the only reason mammary gland tumors were detected at all is because they were visibly obvious.

Effects on mammary growth, lobuloalveolar development, hyperplastic alveolar nodules as well as dysplasias have been detected (Jones and Bern 1977; Jones and Bern 1979) in virgin female BALB/cCrgl mice neonatally treated with estrogen. Mammary tumors have been found in transplantation studies, (Medina 1976) where HANs from DMBA-treated mice were placed into the mammary fat pad of virgin BALB/cCrgl mice. The average time for development (6/6; 100 %) of tumors was 6 months. It is postulated that the mouse mammary tumor virus (MMTV) is essential for the development of mammary gland tumors to develop. This theory is strongly supported by the findings that hormonally neonatally treated mice that have MMTV develop mammary gland tumors (Jones and Bern 1979). It was very unfortunate that the mammary gland was not chosen as a target organ but it was unexpected to find mammary gland tumors in treated inbred mice that lack the MMTV. The induction of mammary gland tumors due to neonatal OH-PCB may be due to the combination of its overall carcinogenicity with its estrogenicity. Future

studies using this animal model are necessary to determine the mechanism of action. In humans, the association of PCBs with breast cancer remains undetermined. Exposure to elevated levels of PCBs is still a potential factor in breast cancer (Laden et al. 2002; Wolff and Toniolo 1995) but a correlation has not been established (Brown 1987; Higginson 1985; Krieger et al. 1994; Laden et al. 2001).

There are two significant results of this study, the first is the demonstration is that hydroxylated PCB congeners are carcinogenic and the second is that the type of CV tract tumors observed in response to treatment with a mixture studies was significantly different than from individual OH-PCBs treatment. For both mixture groups (E2/OH-PCB 30, and OH-PCB 30/61), there were a lower incidence of CV tract "squamous" cell carcinomas and elevated incidence of CV tract "adenosquamous" cell carcinoma. Thus, a shift from squamous to adenosquamous was observed with mice treated with mixtures. This is a very interesting result because it illustrates clearly that the toxic response to mixtures may be different from the toxic response of the individual components of the mixture. Gynecological epithelial tumors are generally grouped into these two major categories based on whether they are derived from mullerian epithelium (adenocarcinoma), or squamous epithelium (squamous cell carcinoma) of the urogenital sinus. The adenosquamous carcinoma of the CV tract may be similar to the adenosquamous carcinoma of the lung that is an example of a heterogeneous tumor (Kanazawa et al. 2000). Adenosquamous carcinoma of the lung and CV tract are similar in a clinical outcome where the prognosis is poorer than patients with either squamous or adenocarcinomas (Farley et al. 2003; Hofmann et al. 1994).

This study supports the hypothesis that neonatal exposure to estrogenic OH-PCBs mimics the ability of E2 to induce CV tract tumors in the BALB/cCrgl mouse. For example, at the higher

dose there was an increase in CV tumors induced by OH-PCB 30 as compared to the lower dose. In addition, similar molecular and morphological effects were true to a lesser extent for PCB-61. The dose of OH-PCB 61 was twice that of OH-PCB 30, therefore, a similar incidence of CV tract tumors was expected based on receptor binding affinities. Instead there was less than half as many; 21 % (5/24), versus 46 % (10/22), incidence rates for CV tract tumors for the high dose of OH-PCB 61 and OH-PCB 30, respectively. This may be a result of toxicity as indicated by higher mortality (Table 2).

Assessing the long-term effects of PCBs is important because the general population is exposed to these chemicals at all stages of human development. In a series of reports, researchers from the Netherlands associated prenatal exposure to PCBs with biological effects (Huisman et al. 1995; Patandin et al. 1999; Patandin et al. 1999). Similarly, perinatal exposure to PCBs is linked to a variety of immunological, neural, and endocrine effects and potentially linked with biological effects on growth, sexual development, and long-term reproductive health (Weisglas-Kuperus 1998). Perinatal exposure to PCBs has been associated with smaller head circumference and lower birthweight (Fein et al. 1984; Taylor et al. 1989). One study also reported a decrease in penis size in boys born to mothers exposed to PCBs, but this finding may be difficult to interpret since the maternal exposure was to a mixture of PCBs most likely contaminated with similar organochlorines, *i.e*., the polychlorinated dibenzo-*p*-dioxins/dibenzofurans (Guo et al. 1995). These studies emphasize the need for testing individual compounds and as compounds in mixtures.

CONCLUSION

In conclusion, OH-PCBs induced predominantly mammary gland and CV tract tumors in mice that were exposed during a critical period of development. OH-PCBs induced tumors in

other organs suggesting the carcinogenic effect is not restricted to estrogen sensitive organs. These findings suggest other organs should be examined in future epidemiological studies with OH-PCBs. Finally, we believe this report is the first to show that a chemical mixture shifts the tumor type from squamous to adenosquamous suggesting that exposure to a mixture may result in the formation of more aggressive tumor type.

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TABLE 1. Chemical nomenclature, abbreviations, and ER alpha binding.

Chemical Name	Abbreviation	C_{50} [a]	Observed log IC_{50} [b]
17 beta-estradiol	E2	1	0.837
2',4',6'-trichlorobiphenyl	PCB 30	-	6.77
2',4',6'-trichloro-4-biphenylol	OH-PCB 30	42	2.84
2',3',4',5'-tetrachlorobiphenyl	PCB 61	-	ND[c]
2',3',4',5'-tetrachloro-4-biphenylol	OH-PCB 61	95	2.15

[a]The molar equivalent required to occupy 50 % of the mouse uterine ER alpha binding site (Korach et al. 1988).

[b]IC_{50}, is the concentration of competitor predicted to cause a 50 % reduction in specific binding of radiolabeled 17β-estradiol to calf uterine ER.

[c]Not Detected (ND) at doses tested (Kramer and Giesy 1999).

TABLE 2. Gross observations from neonatally treated BALB/c mice at 20 months of age.

Neonatal Treatment[a] [µg/pup/day]	DVO[b] (mean ± SE)	Bodyweight (mean ± SE)	Mortality[c] (%)	N[d]
1) Oil	23.8 ± 0.6	25.0 ± 0.37	9	35
2) E2 [5]	10.5 ± 0.4*	23.0 ± 0.43*	16	43
3) E2 [2.5] plus OH-PCB 30 [100]	10.9 ± 0.4*	24.8 ± 0.50	21	24
4) OH-PCB 30 [200]	11.1 ± 0.2*	24.6 ± 0.40	31**	32
5) OH-PCB 30 [20]	24.8 ± 0.4	24.8 ± 0.51	21	39
6) OH-PCB 61 [400]	12.4 ± 0.4*	24.7 ± 0.40	33**	33
7) OH-PCB 61 [40]	17.7 ± 0.8*	25.0 ± 0.44	19	31
8) OH-PCB-30/61 [100+100][e]	12.1 ± 0.4*	25.7 ± 0.62	30**	27
9) OH-PCB-30/61 [10+10][e]	22.4 ± 0.6	25.3 ± 0.33	18	40

[a]Pups were injected subcutaneously within 16 hours after birth for 5 days at the doses indicated using sesame oil as vehicle.

[b]DVO, day of vaginal opening in BALB/cCrgl female mice.

[c]Percent animal dying prior to end of study.

[d]Number of animals used for study.

[e]Equal concentrations of OH-PCB 30 and OH-PCB 61 were used as a mixture.

*P<0.05 versus oil (Tukey-HSD test).

**P<0.05 versus oil (Wilcoxon rank sum test).

TABLE 3. Summary of specific tumor incidence in BALC/c mice treated neonatally and sacrificed at 20 months of age.

Neonatal Treatment	Incidence of Tumor Type									
[µg/pup/day] [a]	ML	H	BA	C	CV	OG	MG	Ot[b]	TNT[c]	N[d]
1) Oil	1	0	0	0	0	0	0	0	1	33
2) E2 [5]	0	0	1	2	16**	1	1	0	18**	37
3) E2 [2.5]/OH-PCB 30 [100]	0	0	0	0	9**	3*	0	1	11*	19
4) OH-PCB 30 [200]	0	0	0	1	10**	3	0	0	12*	22
5) OH-PCB 30 [20]	0	0	3	0	2	0	5	0	9*	33
6) OH-PCB 61 [400]	0	2	0	0	5*	0	1	2	11*	24
7) OH-PCB 61 [40]	2	1	0	0	4*	3	4*	2	15*	30
8) OH-PCB-30/61 [100+100][e]	2	0	1	0	8*	2	0	2	13*	21
9) OH-PCB-30/61 [10+10][e]	0	0	0	0	3	1	3	1	8*	36

ML, malignant lymphoma; H, hemangiosarcoma; BA, bronchoalveolar; C, cholangiocarcinoma of the gallbladder; CV, cervicovaginal tract carcinoma; OG, ovarian granulosa cell tumor; MG, mammary gland carcinoma; Ot, other types of tumors not listed; TNT, total number of tumors found in that treatment group.

[a]Pups were injected subcutaneously within 16 hours after birth for 5 days at the doses indicated using sesame oil as vehicle.

[b]Tumor types that occurred in no more than one animal per group.

[c]Total number of tumors found that treatment group. Some mice had more than one type of tumor.

[d]Number of mice diagnosed by H&E staining.

[e]Equal concentrations of OH-PCB 30 and OH-PCB 61 were used as a mixture.

*P<0.05 versus oil (Fishers exact test).

**P<0.01 versus oil (Fishers exact test).

TABLE 4. Interactive effects on frequency of carcinoma types in the CV tract.

Neonatal Treatment [μg/pup/day] [a]	Total Incidence[b]	Percent frequency	
		Squamous	Adenosquamous
E2 [5]	16/37[c]	41 (15/37)	8 (3/37)
OH-PCB 30 [200]	10/22[c]	36 (8/22)	14 (3/22)
OH-PCB 61 [400]	5/24	13 (3/24)	8 (2/24)
E2 [2.5]/OH-PCB 30 [100]	9/19	16 (3/19)	32 (6/19)*
OH-PCB-30/61 [100+100][d]	8/21	14 (3/21)	24 (5/21)

[a]Pups were injected subcutaneously within 16 hours after birth for 5 days at the doses indicated using sesame oil as vehicle.

[b]Total incidence is the number of CV tract tumors per total number of mice treated.

[c]Note, some mice had more than one type of CV tract tumor.

[d]Equal concentrations of OH-PCB 30 and OH-PCB 61 were used as a mixture.

*$P<0.05$ versus combination of E2 [5] and OH-PCB 30 [200], Fishers exact test.

FIGURE LEGEND

Figure 1. Chemical structures for hydroxylated polychlorinated biphenyls A) 2’4’6’-trichloro-4-biphenylol (OH-PCB 30), and B) 2’3’4’5’-tetrachloro-4-biphenylol (OH-PCB 61).